Automated software-guided identification of new buspirone metabolites using capillary LC coupled to ion trap and TOF mass spectrometry

Application Note

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Abstract

The identification and structure elucidation of drug metabolites is one of the main objectives in in-vitro ADME studies. Typical modern methodologies involve incubation of the drug with subcellular fractions to simulate metabolism followed by LC-MS/MS or LC-MS^n analysis and chemometric approaches for the extraction of the metabolites. The objective of this work was the software-guided identification and structure elucidation of major and minor buspirone metabolites using capillary LC as separation technique and ion trap MS^n as well as electrospray ionization orthogonal acceleration time-of-flight (ESI oaTOF) mass spectrometry as detection techniques.

Buspirone mainly underwent hydroxylation, dihydroxylation and N-oxidation in S9 fractions in the presence of phase I cofactors and the corresponding glucuronides were detected in the presence of phase II cofactors. The use of automated ion trap MS/MS data dependent acquisition combined with a chemometric tool allowed the detection of five small chromatographic peaks of unexpected metabolites that co-eluted with the larger chromatographic peaks of expected metabolites. Using automatic assignment of ion trap MS/MS fragments as well as accurate mass measurements from an ESI oaTOF mass spectrometer possible structures are postulated for these metabolites that were previously not reported in the literature.

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Introduction

In-vitro metabolite identification is being implemented early in the discovery phase to eliminate compounds that produce active or toxic metabolites and therefore would fail later development stages[1]. Different LC/MS techniques are commonly used in metabolite identification. Tandem MS (MS/MS) using a triple quadrupole or an ion trap provides a series of diagnostic fragment ions. During interpretation, the product ion spectrum of the drug can be compared with the product ion spectrum of the unknown metabolite to find common substructures. Multiple stages of MS (MS^n) using an ion trap can provide additional structural information, and can help to decide between different postulated structures. MS^n is also very useful in the structure elucidation of phase II metabolites like glucuronides, whose MS^2 spectra are poor in information and dominated by a very intense neutral loss peak derived from the loss of glucuronic acid. Although MS^n experiments can pinpoint the site of modification very accurately, occasionally a metabolite fragments in a manner that does not provide the required information to unequivocally elucidate its structure. In these cases, the combination of ion trap data and accurate mass measurement with a time of flight (ESI oaTOF) or an hybrid quadrupole-TOF (Q-TOF) instrument may provide the answer by empirical formula calculation[2,3].

Until a few years ago, the only instruments available to perform measurements with highest mass accuracy were the magnetic sector mass spectrometers. Nowadays, ESI oaTOF instruments are also capable of handling this task sufficiently for compound confirmation. This is demonstrated by a comparison study of different types of mass spectrometer instruments for the determination of accurate masses of small molecules[4].Recently, the implementation of oaTOF instruments for the measurement of accurate molecular mass, the calculation of the empirical formula, and consequently the identity confirmation of an unknown compound was demonstrated for the elucidation of the degradation pathway of the widely used antibiotic drug amoxicillin[5-8].

Although LC/MS is robust, sensitive, rapid and easily automated, interpreting the acquired data is typically the largest bottleneck in metabolite identification. Software packages are now available that automatically perform functions that researchers normally complete manually such as the background subtraction of a control data file, identification of unique features between two or more samples and spectral based fragmentation analysis. All of these features help in reducing the data set that the analyst has to examine, thereby increasing throughput[9]. In this Application Note we present a systematic approach for metabolite identification using capillary LC coupled to ion trap MS^n and ESI oaTOF as complementary mass spectrometric techniques as well as the integration of the Advanced Chemistry Development, Inc. (ACD/Labs) software 8.0 release for automated data interpretation.

Experimental

Reagents and supplies

The substance buspirone hydrochloride and the biochemicals beta-nicotinamide adenine dinucleotide phosphate sodium salt (NADP), isocitric dehydrogenase (IDH), uridine 5'-diphosphoglucuronic acid trisodium salt (UDPGA) and adenosine 3'-phosphate 5'-phosphosulfate tetralithium salt tetrahydrate (PAPS) were purchased from Sigma-Aldrich (Taufkirchen, Germany). DL-isocitric acid trisodium salt was purchased from MP Biomedicals GmbH (Eschwege, Germany). Untreated male Sprague Dawley rat liver S9 homogenate (pool of 203) was purchased from XenoTech, LLC (Kansas, USA). All other reagents and organic solvents were of analytical grade and from VWR (Darmstadt, Germany).

Our metabolite identification strategy included the steps shown in Figure 1 and described below.

Incubation of buspirone with rat liver S9 fractions

The incubation mixtures for phase I metabolism consisted of an amount of S9 preparation equivalent to 1 mg protein, 30 µM substrate (buspirone as 1 mg/mL solution in water), 1.2 mM NADP, 2.5 U IDH, 5 mM isocitrate and 5 mM magnesium chloride in 0.1 M phosphate buffer (pH 7.4) made up to a total volume of 1000 µL. The incubation mixtures for phase II metabolism contained additionally UDPGA and PAPS. Incubation was carried out at 37° C for 60 minutes and the reaction was stopped by adding 100 mL perchloric acid and 400 ml acetonitrile followed by centrifugation for 15 min at 14,000 g. The supernatant was evaporated to dryness using a SpeedVac concentrator and reconstituted with water containing 0.1 % formic acid prior to LC/MS analysis as described below. Incubations stopped at 0 min were used as controls.

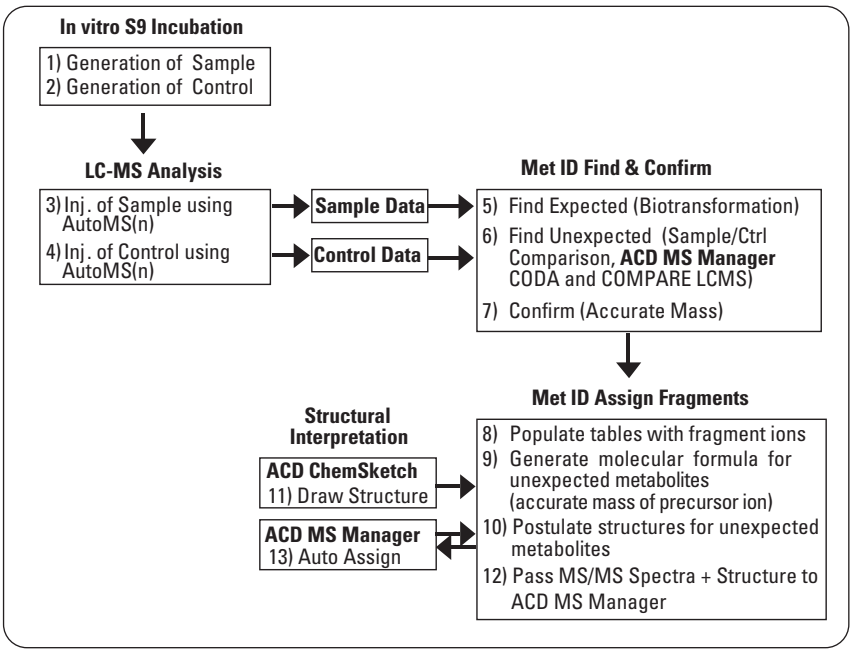

Figure 1
Systematic approach to metabolite identification

Equipment

Capillary liquid chromatography was performed with an Agilent 1100 Series system consisting of a capillary pump, a vacuum degasser and a micro well-plate sampler coupled to an Agilent 1100 Series LC/MSD Trap XCT or an Agilent LC/MSD TOF. The LC/MSD Trap XCT ion trap mass spectrometer was controlled by version 5.3 software. The LC/MSD TOF was controlled by version A.01.01 software.

Chromatographic conditions

The column was a prototype ZORBAX SB-Aq 0.3 x 150 mm, 3.5 µm. The chromatographic conditions were as follows: Solvent A consisted of water containing 0.1 % formic acid and solvent B consisted of acetonitrile containing 0.1 % formic acid. The gradient started with 0 % B, reached 15 % B after 65 minutes, 90 % B after 70 minutes and ended at 80 minutes with a post run time of 10 minutes. The flow rate was 4 µL/min.

Mass spectrometric conditions

The ion trap analysis was performed in positive mode. The drying gas flow was 3.5 L/min, the drying gas temperature was 320 °C, nebulizer pressure was: 50 psi, skim 1 was at 40 V, capillary exit was 100 V and trap drive was 42 V. The Ion Charge Control (ICC) parameters were as follows: target: 200,000, maximum accumulation time: 150 ms and averages: 4. The automatic MS/MS conditions were as follows: number of precursors: 3, active exclusion: on, exclude after 4 spectra, release after 0.3 min, isolation width: 4 m/z, fragmentation amplitude: 1.3 V and CID voltage ramping (Smart-Frag on): 30-200 %.
The ESI oaTOF analysis was performed in positive mode with dual spray for simultaneous introduction of a reference mass solution, because mixing the LC column effluent with a stream of reference material can result in ion suppression, discrimination or adduct formation. To preclude the need for mixing the analyte and the reference compound prior to spray ionization, a dual sprayer interface is used for ESI[10,11].
The drying gas flow was 7 L/min, the drying gas temperature was 300 °C, nebulizer pressure was 15 psi, skimmer was at 60 V, fragmentor was at 150V, capillary was at 5000 V, Scan 50 – 1000 m/z. When operated in this mode with internal reference mass correction, the instrument

routinely produces molecular weight determinations with a mass accuracy within 3 ppm of the true value.

ACD/ Labs software analysis

For the automated identification of metabolites, the ion trap MS and MS/MS data were evaluated with ACD/MS Manager and ACD/ChemSketch from ACD/Labs (Toronto, ON, Canada). The following steps were performed:

1. Generate MS and MS/MS spectra for the metabolized sample and control.
2. Run COMPARELCMS in ACD/MS Manager to find all significant differences between the metabolized sample and the control.
3. Manual inspection of MS/MS spectra of each found metabolite.
4. Propose and draw a structure for each detected metabolite in ACD/ChemSketch.
5. Attach the corresponding hypothetical chemical structure to the MS/MS spectrum.
6. Run *Autoassign* in ACD/MS Manager.
7. The ACD/MS Manager delivers a score for percentage agreement to the MS/MS spectrum of hypothetical metabolite chemical structures.
8. These scores help to decide which is the most likely form for the hypothetical chemical metabolite structure.

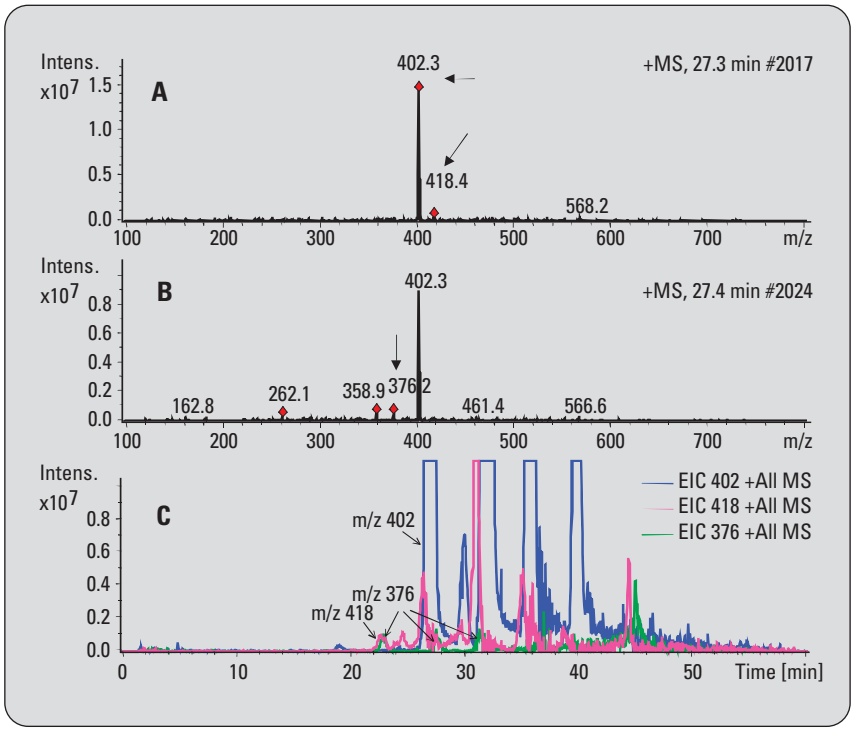

Figure 2
Representation of the data-dependent acquisition feature "N most abundant precursors" in combination with "active exclusion". A) MS spectrum at 27.3 min showing the N most abundant precursors (N=3): m/z 402 and m/z 418 (indicated with a red diamond) for which MS/MS data was collected; B) MS spectrum at 27.4 min showing the actively excluded m/z 402 (no MS/MS data was acquired) and the selection of the N most abundant peaks (N=3): m/z 262, m/z 359 and m/z 376 (indicated with a red diamond) for which MS/MS data were collected; C) overlaid extracted ion chromatograms for m/z 402, m/z 418 and m/z 376 showing the co-elution of the novel metabolites with m/z 376 with the higher level metabolites with m/z 402 and m/z 418.

Results and discussion

Use of ion trap automated data-dependent acquisition

The advanced data-dependent acquisition capabilities of the MSD Trap software were used to increase the amount of unique metabolite data. The "N most abundant precursors" feature determined the number of unique precursor ions from which MS/MS data were acquired. In the present study n=3 was selected. Combined with the "active exclusion" feature, it was especially helpful in acquiring data from less abundant unexpected metabolites in co-eluting peaks. Figure 2 shows how these features automatically identified three novel metabolites (MH^+=376) that co-eluted with the higher level metabolites (MH^+=402 and MH^+=418).

Automated software-guided metabolite identification

The metabolic biotransformation products of buspirone have been well characterized[12,13]. Examples of known buspirone metabolites are hydroxybuspirone (parent + 16), dihydroxybuspirone (parent + 32) as well as the corresponding glucuronides (parent + n * 16 + 176). The metabolites were located by examination of expected MH^+ values in the MS traces as well as examination of diagnostic fragments in the MS/MS spectra[13]. According to Zhu et al.[13], these fragments are m/z 168 (A), m/z 219 (B), m/z 180 (C), m/z 222 (D), m/z 150 (E), m/z 265 (F), m/z 122 (G) and m/z 291 (H) for buspirone as shown in the figure 3. Moreover the COMPARELCMS algorithm within ACD/MS Manager was invoked to find all significant differences between the sample and the control without regard to an expected m/z value. After this processing, the result was a display of unique mass chromatograms from the comparison as shown in figure 4. Some differences between the total ion chromatogram of the control (A) and the total ion chromatogram of the incubated sample (B) were obvious at retention times between 25 and 40 minutes but the display of unique mass chromatograms showed the presence of a variety of metabolites that were not obvious in the total ion chromatogram of the incubated sample and could have been missed during a manual inspection. The peaks identified by the

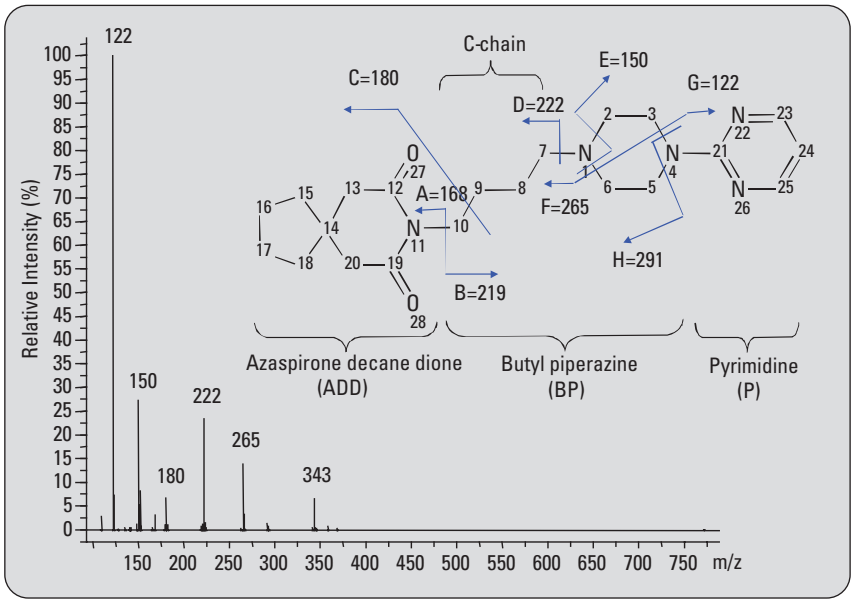

Figure 3
MS/MS spectrum and representation of fragment ions of buspirone according to Zhu et al[13].

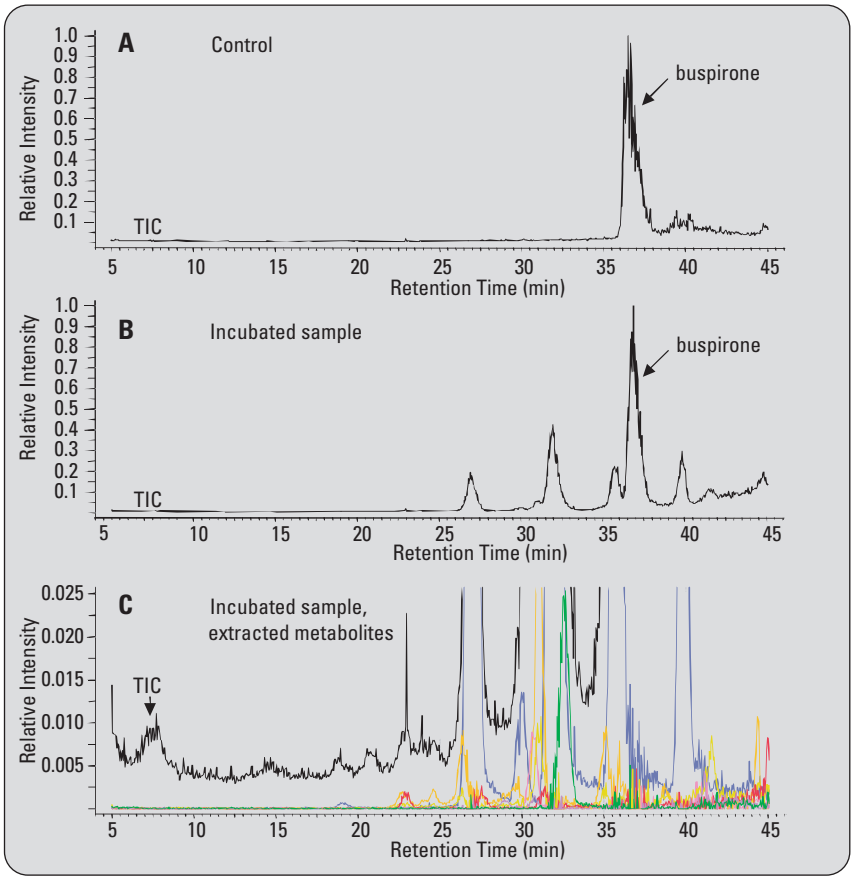

Figure 4
Total ion chromatograms for the control (A) and the incubated sample (B). A display of unique mass chromatograms from the comparison of the control and the incubated sample is shown in (C).

COMPARELCMS algorithm as unique to the incubated sample were further investigated. Potential metabolite structures were assigned with the knowledge of possible metabolite transformations from the literature[12,13], the available MS^2 spectra, and automatic assignment of spectral fragments using the autoassignment module within ACD/MS Manager. Single hydroxylations, double hydroxylations, buspirone N-oxide, as well as the corresponding glucuronides previously reported in the literature were detected. Additionally five unexpected metabolites were found, namely a metabolite with MH^+=360, three metabolites with MH^+=376 and a metabolite with MH^+=400 (table 1). For the metabolite with MH^+=360, two possible structures were hypothesized (a N,N-desethyl structure and a decarbonylated structure, figure 5) and the ACD MS/Manager software was used to support the assignment. The auto assignment module within ACD MS/Manager fragments the proposed structure according to accepted rules and forms a table of fragments containing correlations between fragments and mass values. In this manner the N,N-desethyl structure was determined to be the preferred one, showing a higher percentage of spectrum assigned as shown in figure 5.

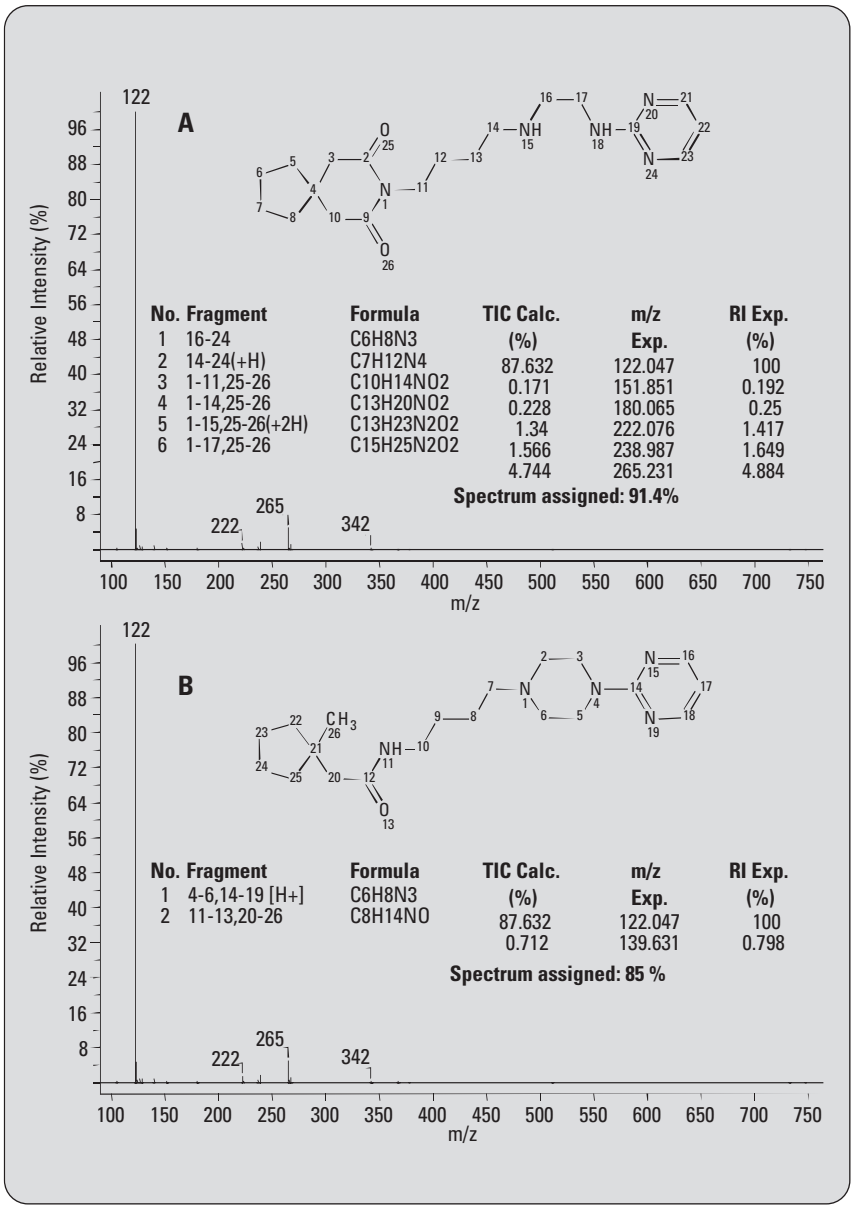

Figure 5

MS/MS spectrum for the novel metabolite with m/z 360 at 32.7 min. Two possible structures were postulated. Automatic assignment of MS fragments using the ACD/MS Manager software suggests structure A, with 91.4 % of the fragments in the spectrum assigned, is preferred over structure B with only 85 % of the fragments assigned (assignment options: mass range from 1 to 360, relative abundance range from 1 to 100).

Metabolite (M+H)+	Retention time [min]	Postulated structure	Fragment ions A	B	C	D	E	F	G	H
402	18.8	Hydroxybuspirone (ADD group) *		219		238	150	281	222	
376 (unexpected)	22.7	N,N-desethyl hydroxybuspirone				238	122	281	--	281
418	23.0	Dihydroxybuspirone (ADD group) *		219		254	150	297	122	323
418	24.5	Dihydroxybuspirone (ADD group) *	200	219	212	254	150	297	122	323
594	26.1	Dihydroxybuspirone Glucuronide [MS3 (594 → 418)]					166	281	138	
400 (unexpected)	26.1	Oxo-Buspirone (ADD group)*		219		236	150	279	122	
418	26.2	Dihydroxybuspirone (ADD and P groups) *	184	235	196	238	166	281	138	
402	26.7	Hydroxybuspirone (ADD group) *		219	196	238	150	281	122	307
376 (unexpected)	27.4	N,N-desethyl hydroxybuspirone				238	122	281	--	281
418	29.1	Dihydroxybuspirone (C-chain and P groups) *	168	235	180	238	166	281	138	307
402	30.1	Hydroxybuspirone (ADD group) *		219	196	238	150	281	122	
578	30.2	Hydroxybuspirone Glucuronide [MS3 (578 → 402)]	168	235	180	222	166	265	138	291
376 (unexpected)	31.3	N,N-desethyl hydroxybuspirone	168			222	138	265	--	265
360 (unexpected)	32.0	N,N-desethyl buspirone (BP group) *			180	222	122	265	--	265
402	32.7	Hydroxybuspirone (ADD group) *		219		238	150	281	122	307
418	34.6	Dihydroxybuspirone	168		196	238		281	122	
402	35.3	Hydroxybuspirone (P group) *	168	235	180	222	166	265	138	291
418	36.2	Dihydroxybuspirone		219		238		281	122	323
386	36.7	Buspirone				222	150	265	122	
402	39.9	Buspirone N-oxide	168		180	222		265	122	291

Table 1

Identified metabolites, postulated structures and observed MS/MS fragment ions. A blank entry means "not found", and a "--" entry signifies "not applicable". The side of modification is given when possible and denoted by ( ) *. Originations of fragment ions as well as abbreviations for the buspirone substructures are given in figure 3.

Two of the metabolites with MH^+=376 produced MS/MS spectra where the fragments D, F and H shifted in mass by 16 u from those observed in the MS/MS spectrum of the metabolite with MH^+=360, indicating that these metabolites are hydroxylated forms of the N,N-desethyl buspirone metabolite and, that the hydroxyl group is present in the azaspirone decane dione substructure or in the C-chain. The third metabolite with MH^+=376 produced a MS/MS spectrum where only fragment E shifted in mass by 16 u from that observed in the MS/MS spectrum of the metabolite with MH^+=360, indicating that this metabolite is also an hydroxylated form of the N,N-desethyl structure and that the hydroxyl group is present in this case in the piperazine or in the pyrimidine ring (table 1, figure 3). The metabolite with MH^+=400 produced a MS/MS spectrum where the fragments D and F shifted in mass by 14 u from those observed in the MS/MS spectrum of buspirone, indicating that this metabolite is either a ketone or a methylated metabolite of buspirone and, that the modification is present in the azaspirone decane dione substructure (table 1, figure 3). For further confirmation of the postulated new buspirone metabolite structures ESI TOF data was acquired for empirical formula calculation.

Confirmation of postulated structures using TOF analysis

In the software guided analysis of the ion trap data of phase I and phase II buspirone metabolites five unexpected metabolites were detected, namely a metabolite with m/z 360 together with its hydroxylation products as well as a new metabolite with m/z 400. Structures for these unexpected metabolites were postulated and confirmed by an ESI oaTOF analysis that allowed the calculation of the empirical formula from the obtained high accurate mass measurement. This was especially useful for the metabolite with m/z 360 (confirmed N,N-desethyl structure) and for its hydroxylation products because there was another possible structure for these metabolites obtainable from a decarbonylation reaction. The exact mass for the protonated N,N-desethyl structure (figure 5A, $C_{19}H_{30}N_5O_2$) is calculated at m/z 360.2400, for the possible protonated decarbonylated structure (figure 5B, $C_{20}H_{34}N_5O$) the exact mass is calculated to m/z 360.2763. The diffrence between values, 0.036 Da or ca. 100 pm, is much larger than the 3-ppm error routinely obtained on this instrument, so these possibilities may be distinguished using ESI oaTOF. After ESI TOF analysis and extraction of the obtained TIC using an appropriate mass range only the molecular ion at m/z 360.2398 was detected. This is a mass difference of -0.2 mDa to the proposed N,N-desethyl metabolite giving a mass error for the proposed empirical formula of 0.4 ppm. This clearly confirms the identity of this N,N-desethyl metabolite (figure 6).

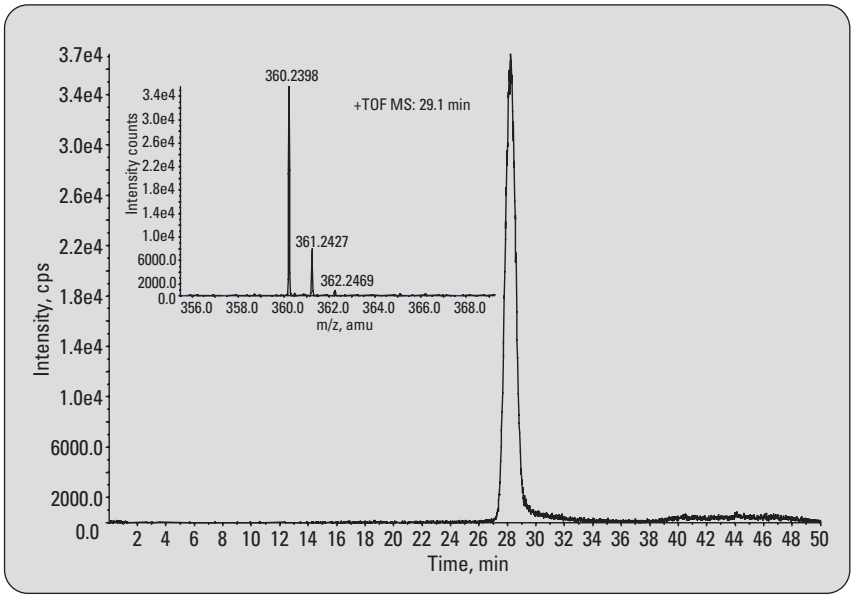

Figure 6
ESI oaTOF detection of N,N-desethyl buspirone metabolite, MH+=360.2400, $C_{19}H_{30}N_5O_3$, measured mass: 360.2398, mass accuracy: 0.4 ppm; mass difference –0.2 mDa.

Additionally, the correspondent hydroxylated buspirone metabolites of the N,N-desethyl metabolite ($C_{19}H_{30}N_5O_3$, m/z 376.2349) were isolated from the total ion chromatogram. Only the possible metabolites with the measured m/z 376.2344 were found with –0.5 mDa mass differences and with a mass error of 1.2 ppm. This additionally confirms the structure of the new buspirone N,N-desethyl metabolite.The new buspirone metabolite with MH^+=400, which was identified in the ion trap analysis, could also be confirmed by means of the ESI oaTOF. Two possible structures were postulated, namely a ketone metabolite and a methylated metabolite. The empirical formula of the postulated ketone metabolite (oxobuspirone, $C_{21}H_{30}N_5O_3$) gives a calculated mass at m/z 400.2346 and the empirical formula of the methylated metabolite ($C_{22}N_5O_2H_{33}$) gives a calculated mass at 400.2634. After ESI TOF analysis and extraction of the obtained TIC by an appropriate mass range only the molecular ion at m/z 400.2349 was detected, which is a mass difference of 0.3 mDa and a respective mass error of -0.6 ppm. This measured highly exact mass confirms this new oxobuspirone metabolite with high confidence.

Identified metabolites using ion trap and TOF analysis

A summary of the postulated metabolites is shown in table 1.

Conclusions

For metabolite identification, the present approach provides sensitive capillary LC coupled to mass spectrometry using an ion trap to produce MS/MS and MS^3 product ion spectra as well as TOF accurate mass data to confirm the postulated structures. The metabolites could be easily detected using ion trap intelligent data-dependent acquisition. The metabolite spectra were easily extracted using chemometric tools and their structures elucidated using the combination of ion trap and TOF data. The majority of the known buspirone metabolites could be detected as well as five unexpected metabolites not previously reported in the literature. All postulated metabolite structures could be additionally confirmed by exact mass measurement by means of an ESI TOF and empirical formula calculation.

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